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Repeat Sequences in Block 2 of *Plasmodium falciparum* Merozoite Surface Protein 1 Are Targets of Antibodies Associated with Protection from Malaria

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Human antibodies to the block 2 region of *Plasmodium falciparum* merozoite surface protein 1 (MSP1) are associated with a reduced prospective risk of clinical malaria. Block 2 is highly polymorphic, but all known alleles can be grouped into three major types. Two of these types (the K1-like and MAD20-like types) contain type-specific sequences (found in all alleles of a particular type) that flank polymorphic tripeptide repeats. These repeats contain both type-specific and subtype-specific sequences. To evaluate the antibody recognition of these parts of block 2, a new panel of six recombinant proteins was used (fused type-specific flanking sequences and two representative repeat sequences for each of the K1-like and MAD20-like types separately). Extensive testing of these antigens and full-length block 2 antigens showed that human serum immunoglobulin G antibodies induced by infection can recognize (i) type-specific epitopes in the repeats, (ii) subtype-specific epitopes in the repeats, or (iii) type-specific epitopes in flanking sequences. A large prospective study in The Gambia showed that antibodies to the repeats are strongly associated with protection from clinical malaria. The results are important for design of a vaccine to induce protective antibodies, and they address hypotheses about repeat sequences in malaria antigens.

Merozoite surface protein 1 (MSP1) is the most abundant protein on the surface of the invasive blood stage form of malaria parasites and is a leading candidate for a vaccine against *Plasmodium falciparum* malaria (18). It exists as a non-covalently linked complex of four fragments (83, 28, 38, and 42 kDa) generated by proteolytic cleavage of a ~190-kDa precursor that is membrane anchored by glycosylphosphatidylinositol at the C terminus. The primary structure of MSP1 is polymorphic, and ~40% of the amino acid residues are different in different allelic forms in *P. falciparum* (24, 33).

Immunization with MSP1 has been performed with experimental primate challenge models (*Aotus* and *Saimiri* models). Early studies demonstrated that significant protection from *P. falciparum* parasite challenge was induced by the whole ~190-kDa MSP1 or a large portion of the sequence (19, 31). Subsequent studies have shown that some protection can be induced by immunization with recombinant proteins representing a C-terminal 42-kDa fragment (13) or the cleavage product MSP1₁₉ (22). Naturally acquired human antibodies to MSP1₁₉ have been associated with a reduced risk of clinical malaria in several studies (1, 8, 14, 16, 29), although not in all studies (15). Some monoclonal antibodies to MSP1₁₉ can inhibit merozoite invasion in vitro (3, 4, 35), and a recent study has shown that a substantial proportion of all naturally acquired human antibodies which inhibit merozoite invasion are

targeted to MSP1₁₉ (25). Fewer studies have focused on the rest of the MSP1 molecule (12, 17, 34), although the N-terminal block 2 region has been found to be under the strongest natural selection pressure (14), and antibodies specific for common allelic types of block 2 are strongly associated with a reduced risk of clinical malaria (14). Moreover, a monoclonal antibody against block 2 inhibits parasite growth in vitro (23).

The polymorphic block 2 region of MSP1 can be classified into three main sequence types, K1-like, MAD20-like, and RO33-like, which range in size from 44 to 89 amino acids (24). The K1-like and MAD20-like types contain different tripeptide repeat sequences with serine at the first position, and variations in the sequence and number of repeats produce subtype differences within each of these types. The repeats are flanked by type-specific nonrepetitive sequences (24). Analyses of the specificities of acquired human antibodies with different full-length block 2 recombinant proteins have identified type- and subtype-specific antibodies in sera from individuals in malaria-endemic areas of Sudan, The Gambia, and Kenya (10–12, 14). Antibodies from individuals in Senegal have also been shown to react with a variety of synthetic peptides based on block 2 sequences (20). Understanding the importance of repeat sequences is of particular interest, as repeats are present in many different *P. falciparum* antigens (36). Although it has been suggested that repeats might be detrimental to the development of protective immune responses (2, 9, 30), high levels of antibodies against the repeats in the circumsporozoite protein vaccine antigen are associated with protection from experimental or natural challenge infection in vaccinees (5, 21).

In this paper, recombinant proteins consisting of the MSP1

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block 2 repeat sequences from four alleles (two representatives of the K1-like type and two representatives of the MAD20-like type) and proteins consisting of the nonrepeat flanking sequences of each of these two types are described. The four repeat sequence alleles chosen for analysis are the alleles for which there are existing full-length block 2 antigens and for which the exact corresponding parasite lines can be cultured (10). Mice were immunized with these antigens, and the specificities of antibodies against parasites belonging to different strains were assayed. The antibody specificities in African adult sera were determined with these antigens and full-length block 2 antigens, and sera from a large cohort of children were then studied to identify targets of antibodies associated with protection from malaria.

MATERIALS AND METHODS

Design and construction of MSP1 block 2 repeat and flanking sequences for cloning and expression. Six new recombinant proteins (Fig. 1) were designed; four of these proteins represented repeat sequences, and two represented fused flanking sequences of the K1-like and MAD20-like types of MSP1 block 2. DNA sequences for cloning were produced by using the Expand High Fidelity PCR system (Roche, East Sussex, United Kingdom) with synthetic oligonucleotide primers, as follows. For the fused K1 flanking sequence, a single-stage PCR was performed with HB3 genomic DNA as the template and primers K1flankmutF1 (5'-GGATCCAATGAAGAAGAAATTACTACAAAAGGTGCAGGCGCCA GTC-3') and K1flankR1 (5'-GAATTCCTTAGCTGCATCAGCTGGAGG-3'), which removed all repeat motifs from the PCR product. For the fused MAD20 flanking sequence, two stages of PCR were performed; the first stage produced fragments *MAD20fg1* and *MAD20fg2*, which were amplified from genomic DNA (Wellcome isolate of *P. falciparum*) by using primer MAD20flankF1 (5'-GGATCCAATGAAGGAACAAGTGAAC-3') plus primer MAD20flankR1 (5'-TTACCTGAGGCGGCCACCTTTGAACCAAGGTGTACT-3') and primer MAD20flankF2 (5'-CAAAGGGTGGCGCCTCAGGTAATTCAAGCAGTAC AA-3') plus primer MAD20flankR2 (5'-TGAATTCCTTCATTGAATTATCTG AAGG-3'). The two fragments were isolated by gel purification and mixed at an equimolar ratio for a second stage of amplification, in which the fragments were fused via complementary overhangs in MAD20flankF2 and MAD20flankR1. The 3D7 and Palo Alto repeats were produced synthetically with two stages of PCR. The product of the first-stage PCR was extended by using complementary primers. For the 3D7 repeats the first-stage PCR was performed with complementary primers 3D7repF1 (5'-GGATCCGGCGCCAGTGGCGAGAGTGGC GCAAGTGGCGAGAGTGGCGCAAGTGCTCAAAGTGGTGCATCCGCT CAATCC-3') and 3D7repR1 (5'-AGACGGACCGCTTGTACCGGACTGCG CACTCGACCACTCTGGGCACTTGCACCGATTGACGCGGTGCACC ACTTTG-3'), and the product was gel purified and then mixed with an equimolar amount of 3D7repR2 (5'-GAATTCCTTAGGCGCCTGTACCGGACTGCG CACTCGACCACTCTGGGCACTTGCACCGATTGACGCGGTGCACC ACTTTG-3') for the second-stage amplification. For the Palo Alto repeats the first round of PCR was performed with the complementary primers PaloAltoF1 (5'-GGATCCGGCGCCAGTGGTCAAAGCGGCACAAGCGGC ACAAGCGGCACAAGTGGTACAAGTGGTACAAGTGGTACAAGCGGT A-3') and PaloAltoR1 (5'-GCACCTGTACCACTTTGACCACTTTGAGC ACTTGTGCACTGGTGGCGCTCTGACGCTTGTACCGCTTGTACCA TTTGTACCACTTGTACCACTTG-3'), and the product was gel purified and mixed with an equimolar amount of PaloAltoR2 (5'-GAATTCCTTAGGCG

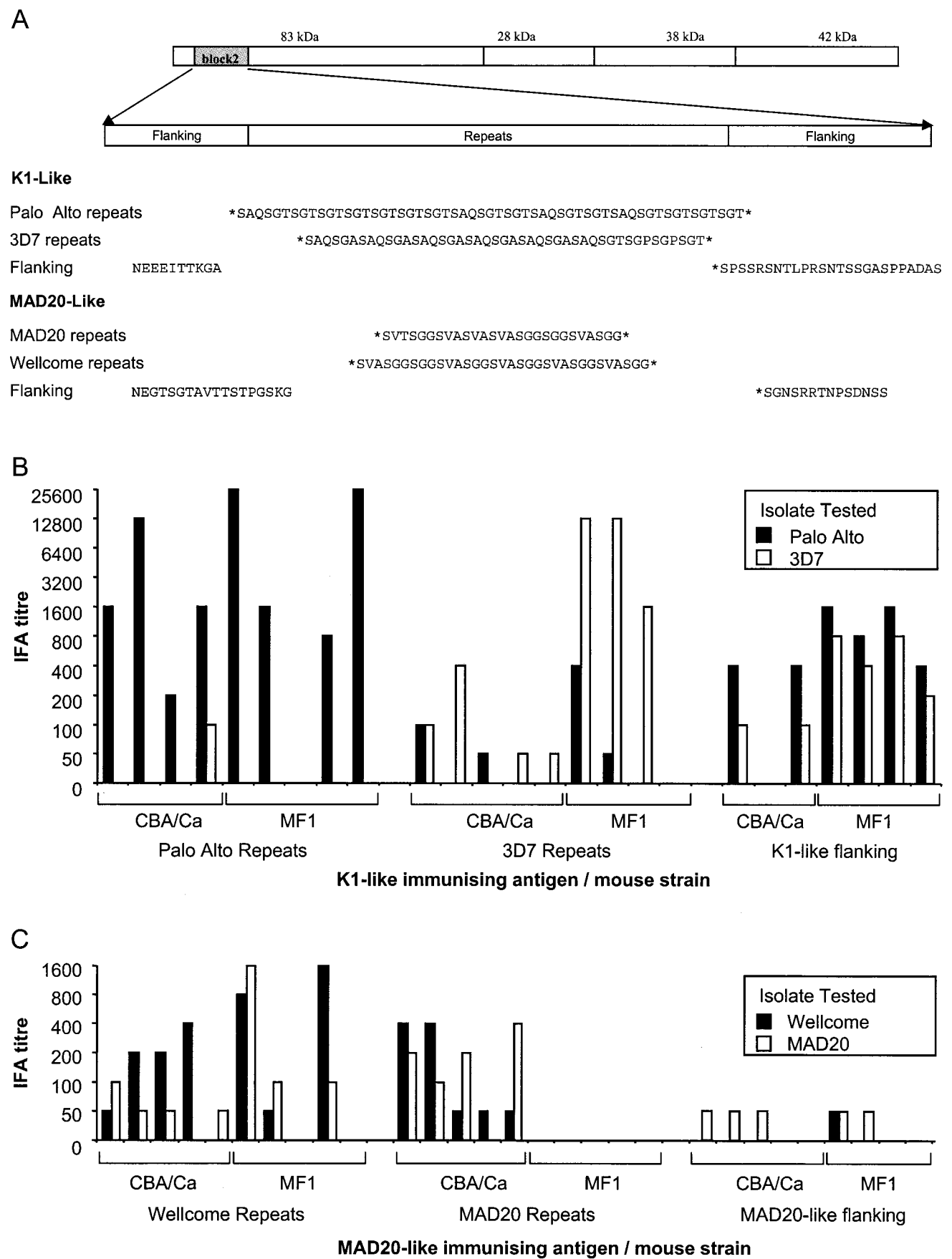
CCTGTACCACTTGTACCACTTGTACCACTTGTACCACTTTGAGCACT TGTACCACTTGTACCACTTTGAGC-3') for the second-stage PCR. The MAD20 and Wellcome repeats were produced synthetically by PCR elongation by using the following complementary primer pairs: MAD20repF1 (5'-GGATC CGGCGCCTCTGTTACTTCAGGTGGTTCAGTTGCTTCAGTTGCTTC GTTGCTTCAGGTGGTAGC-3') plus MADrepR1 (5'-GAATTCCTTAGGCG CCGCGCCTGAAGCAACTGAGCCACCGCTACCACTGAAGCAACGG AGGC-3') and WellcomerepF1 (5'-GGATCCGGCGCCTCAGTTGCTTCAG GTGGTTCAGGTGGCTCAGTTGCTTCAGGTGGCTCAGTTGC-3') plus WellcomerepR1 (5'-GAATTCCTTAGGCGCCACCACTGAAGCTACCGAG CCGCGGAAGCTACCGAGCCGCGGAAGCAACTGAGCCACTGA GCAACTGA-3'), respectively.

Production and purification of recombinant proteins. All DNA products were cloned into the PCR cloning vector pGEM T (Promega, Southampton, United Kingdom), and transformations were done in *Escherichia coli* competent strain JM109 before sequencing to ensure the fidelity of each construct. Each coding region was excised with *Bam*HI and *Eco*RI by using sites engineered into the primers before cloning into the complementary sites of pGEX-2T (32). Cloned recombinant plasmids were sequenced to ensure complete fidelity and then used to transform *E. coli* host strain BL21 for optimal expression of the recombinant proteins, which was achieved after 4 h of incubation with 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Bacterial sonicates were prepared, and this was followed by purification of glutathione *S*-transferase (GST) fusion proteins by using glutathione Sepharose beads in bulk pack columns (Amersham Pharmacia Biotech). Purified fractions of each recombinant protein were pooled, the concentrations were determined by the Protein Assay (Bio-Rad Ltd.), and the preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Immunization of mice and assays of murine antibodies. Outbred MF1 and inbred CBA/Ca mice were immunized with the new block 2 recombinant proteins by using a protocol similar to that described elsewhere (10). Briefly, each purified block 2 fusion protein was diluted with phosphate-buffered saline (PBS) to a concentration of 1 mg ml⁻¹, and 3 volumes of Inject Alum (Perbio Science UK, Cheshire, United Kingdom) was added and allowed to mix for 30 min at room temperature. The antigen-adjuvant mixture was administered intraperitoneally; five MF1 and five CBA/Ca mice were immunized with each protein (50 μ g per dose in a final volume of 200 μ l). Three doses were administered at monthly intervals, and blood was collected before immunization and 12 to 14 days after each dose. The specific antiparasite reactivities of all sera from the final bleed were assessed by performing an immunofluorescence assay (IFA) as described previously (10). Briefly, multiwell slides of schizonts were prepared from cultured *P. falciparum* strains Palo Alto, 3D7, Wellcome, and MAD20; the purity and identity of each strain were confirmed by PCR and sequencing of MSP1 block 2 from genomic DNA; and slides were acetone fixed and air dried. Six serial doubling dilutions of the murine sera (1/50 to 1/1,600) in PBS with 1% bovine serum albumin (20 μ l/well) were tested. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch) was used as the second antibody at a 1/200 dilution, and slides were mounted in Vectorshield mounting medium with 4',6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, Calif.) and examined with a fluorescence microscope at a magnification of \times 1,000. The endpoint titer of each serum was defined as the highest dilution that resulted in bright and clear schizont-specific fluorescence; sera that were positive at a dilution of 1/1,600 were retested at higher dilutions to determine their endpoint titers.

Reactivities of recombinant proteins with human antibodies. Sera from 38 adults living in malaria-endemic areas of Lagos, Nigeria, were assayed for IgG reactivity to MSP1 block 2 recombinant antigens. The adults (ages, 18 to 60 years) each gave informed consent to donate a 20-ml venous blood sample while accompanying their children at the Massey Street Children's Hospital, Lagos

FIG. 1. (A) Schematic representation of MSP1 block 2 repeat and flanking sequence fusion proteins used in this study. Primary amino acid sequences are shown, and they start immediately downstream of the GST fusion partner sequence (the *Bam*HI site was used to clone the inserts into PGEX-2T). Note that each flanking sequence construct is a fusion of the left and right flanking sequences. The asterisks represent *Nar*I sites (which encode a glycine and an alanine) created to allow the sequences to be rearranged in a modular format for further work. (B) Titers, as determined by IFA (against *P. falciparum* schizonts), of serum IgG in mice (CBA/Ca and MF1 strain individuals) immunized with each of the K1-like block 2 repeat antigens or flanking sequence antigen. The reactivities against parasites with K1-like block 2 alleles (isolates Palo Alto and 3D7) are shown; the reactivities against parasites with MAD20-like block 2 alleles (isolates Wellcome and MAD20) were negative. (C) Titers, as determined by IFA (against *P. falciparum* schizonts), of serum IgG in mice (CBA/Ca and MF1 strain individuals) immunized with each of the MAD20-like block 2 repeat antigens or flanking sequence antigen. The reactivities against parasites with MAD20-like block 2 alleles (isolates Wellcome and MAD20) are shown; the reactivities against parasites with K1-like block 2 alleles (isolates Palo Alto and 3D7) were negative.



Island; approval of the ethical committee of the National Institute for Medical Research, Nigeria, was obtained. The adults did not have malaria at the time of sampling (July and August 1997), but all of them reported a previous history of malaria, and a high proportion had antibodies to *P. falciparum* antigen EBA-175 (26). The antigens used included the six novel recombinant proteins listed above plus the four corresponding full-length block 2 constructs (of the K1-like and MAD20-like types) characterized previously: MAD20, Wellcome, Palo Alto, and 3D7 (10–12). The allele frequencies of the K1-like and MAD20-like types of MSP1 block 2 in a nearby area of southwest Nigeria have been described to be 0.52 and 0.29, respectively (14). The sera were tested in duplicate with each antigen by using a protocol described previously (10). Briefly, 50 ng of recombinant antigen per well was coated overnight at 4°C in 100 µl of coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.3) onto Immunolon 4HBX flat-bottom microtiter plates (Dynex Technologies Inc). The plates were washed (in PBS with 0.05% Tween 20), blocked (with 1% skim milk) for 5 h, and washed again. Sera were diluted 1/500 in blocking buffer, and duplicate 100-µl aliquots were incubated overnight at 4°C in antigen-coated wells. The wells were washed and then incubated with 100 µl of horseradish peroxidase-conjugated rabbit anti-human IgG (at a dilution of 1/5,000) (Dako Ltd.) before detection with *O*-phenylenediamine-H₂O₂ (Sigma). The mean optical density of each serum-antigen reaction mixture was calculated after correction for binding of the serum to GST alone (the background optical density was generally <0.1). A serum was scored positive if the corrected optical density was higher than the mean plus 3 standard deviations of values for 20 negative control sera from United Kingdom residents who had never had malaria. By using selected positive sera, competition enzyme-linked immunosorbent assays (ELISAs) for pairs of antigens were carried out to define the specificities of the antibodies. The protocol was identical to that used for direct ELISAs except that sera diluted 1/500 were preincubated with soluble competing antigens at concentrations ranging from 0 to 10 µg ml⁻¹ for 5 h before incubation overnight with 50 ng of plate-bound antigen.

Prospective study of antibody reactivities and incidence of malaria in The Gambia. A cohort of 334 Gambian children (ages, 3 to 7 years) were monitored for clinical malaria by active and passive case detection throughout the annual malaria transmission season in 1996 (from June to November). Sera were collected in June for analysis of antibody reactivities prior to this. The subjects or their guardians gave informed consent, and the study was approved by the Scientific Co-ordinating Committee of the Medical Research Council in The Gambia and the Joint Ethical Committee of the Medical Research Council and Gambian Government. Clinical malaria was defined as a *P. falciparum* parasitemia of >5,000 parasites µl⁻¹ occurring at the same time as a febrile episode with an axillary temperature of >37.5°C, the definition normally used in studies of endemic malaria in West Africa (14, 15, 29). This was the same cohort previously studied for antibodies to full-length block 2 antigens (14) except that three sera that were tested previously were no longer available. The allele frequencies of the K1-like and MAD20-like types of MSP1 block 2 in this area have been described to be 0.51 and 0.33, respectively (14). Sera were tested in duplicate by ELISAs to determine the reactivities with the six new MSP1 block 2 recombinant proteins (MAD20 repeats, Wellcome repeats, 3D7 repeats, Palo Alto repeats, MAD20-like flanking sequence, and K1-like flanking sequence). The mean optical density was corrected for binding to GST, and positive results were scored by using the criteria outlined above. ELISA results were imported into an EXCEL worksheet by investigators blinded from the epidemiological data (kept separately on a STATA file). The data files were merged, and after this univariate and multivariate statistical analyses were performed with EPI-INFO, version 6, and SPSS, version 9.0.

RESULTS

Characterization of the MSP1 block 2 repeat and flanking sequence antigens. The six new recombinant GST fusion proteins (Fig. 1A) were expressed as single products of the expected size after purification, as determined by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Groups of mice (strains CBA/Ca and MF1) were immunized with each of these proteins, and their specific antibody reactivities against schizonts of different *P. falciparum* isolates were determined by IFA endpoint titration (Fig. 1B and C). The Palo Alto repeat and 3D7 repeat antigens (both K1-like type) induced antibodies specific for Palo Alto and 3D7 parasites, respectively (i.e., predominantly subtype-specific antibodies);

the mice immunized with the Palo Alto repeats had higher titers against the Palo Alto parasites than against the 3D7 parasites ($P = 0.012$, as determined by the Wilcoxon signed rank test), and the mice immunized with the 3D7 repeats had higher titers against the 3D7 parasites than against the Palo Alto parasites ($P = 0.041$, as determined by the Wilcoxon signed rank test) (Fig. 1B). The K1-like flanking antigen induced type-specific antibodies against both these K1-like parasites (Fig. 1B). None of the K1-like antigens induced antibodies to MAD20-like parasites or vice versa. Immunization with the Wellcome repeat antigen or the MAD20 repeat antigen induced antibodies in most mice that reacted with both the Wellcome and MAD20 parasites (a predominantly type-specific response); the titers to the Wellcome and MAD20 parasites were not significantly different in each of these groups ($P > 0.2$ for each comparison, as determined by the Wilcoxon signed rank test) (Fig. 1C). Mice immunized with the MAD20 flanking region protein were negative or had very low reactivities (Fig. 1C). The broad type specificity of antibodies reactive to MAD20-like repeat antigens contrasts with the predominant subtype specificity of antibodies to K1-like repeat antigens.

Specificity of human serum IgG to MSP1 block 2 full-length, repeat, and flanking sequence antigens. Thirty-eight adult Nigerian sera were tested against the new block 2 repeat and flanking sequence antigens and against the corresponding full-length block 2 antigens previously described (10). Eighteen (47%) of the 38 sera were positive with one or more of the antigens as determined by ELISA. The specificities of antibodies to the K1-like and MAD20-like antigens are shown in Fig. 2 and 3, respectively.

Fifteen of the 38 sera contained antibodies against the full-length K1-like block 2 antigens (10 sera recognized both full-length Palo Alto and 3D7 antigens, and 5 sera recognized only the Palo Alto antigen) (Fig. 2A). As expected, sera specific for the Palo Alto antigen reacted with epitopes within the repeat sequences (positive for the Palo Alto but not the 3D7 repeats). Of the sera that showed broader specificity, four recognized the K1-like flanking sequence antigen, and four others recognized both Palo Alto and 3D7 repeats. There were differences in the levels of antibodies to the Palo Alto and 3D7 antigens in the latter four sera, although they were positive for both. Figure 2B shows the specificity of antibodies in serum N1, as determined by a competition ELISA, which recognized the Palo Alto repeats strongly and the 3D7 repeats very weakly. In contrast, serum N20 had a predominant reactivity to 3D7 repeats and weaker reactivities to Palo Alto repeats and epitopes on full-length antigens (Fig. 2C). Other sera also showed differences in the antibody reactivities to the Palo Alto and 3D7 repeats, and no serum contained antibodies that were able to bind these two sequences similarly. Thus, antibodies to these two K1-like repeats were mostly subtype specific.

In contrast, the antibody reactivities to the block 2 MAD20-like antigens exhibited relatively broad type specificity. Ten sera tested positive with one or more of the antigens (Fig. 3A), and seven of these sera recognized both full-length block 2 antigens. Antibodies in these sera reacted with the repeats. When sera were analyzed by competition ELISA, it was apparent that the majority of the reactivity was due to antibodies which recognized both MAD20 and Wellcome repeats, although there were some detectable differences in the abilities

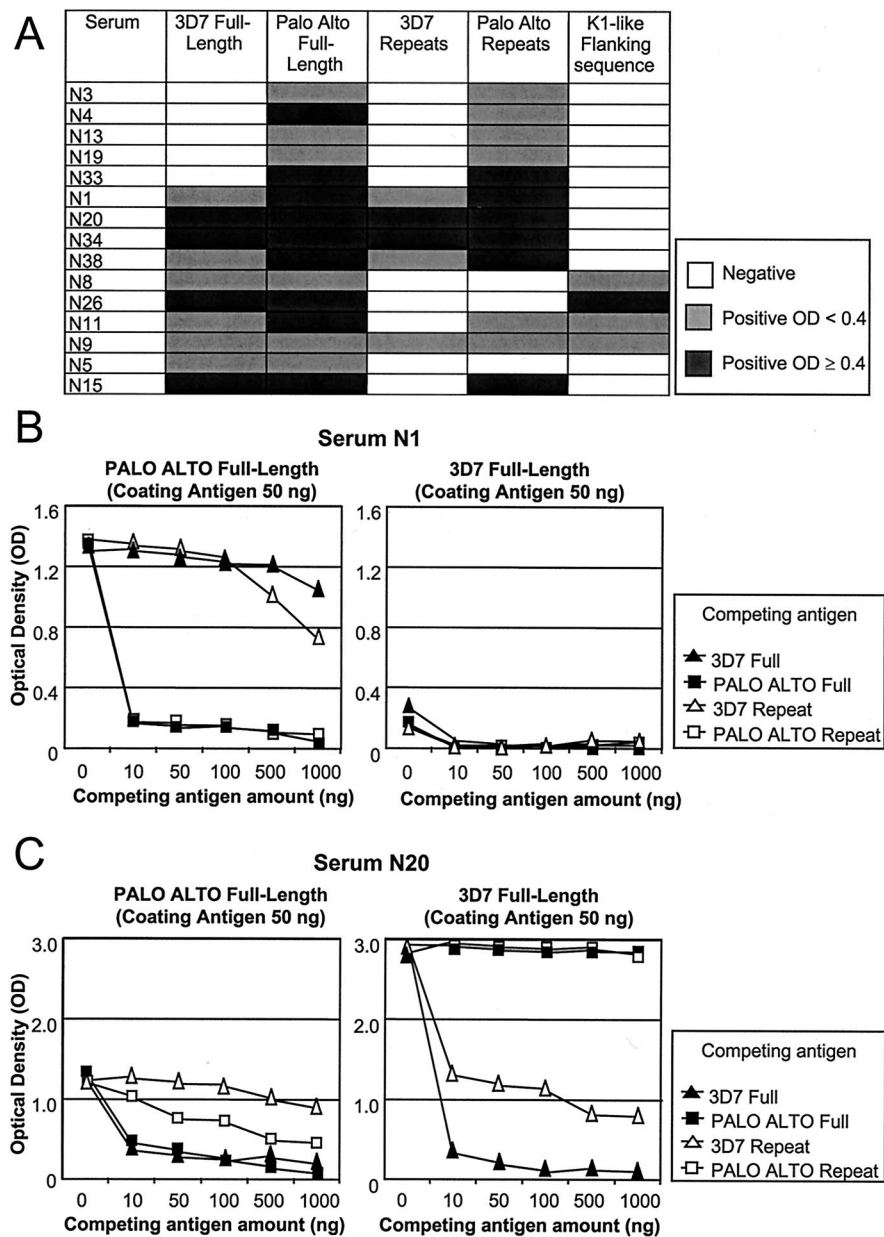


FIG. 2. Reactivities as determined ELISA of Nigerian adult sera with K1-like antigens. (A) Reactivities of all sera which were positive for one or more K1-like antigens. Positive ELISA optical density (OD) values are the values above the cutoff defined for each antigen by the mean plus 3 standard deviations for negative control sera (the cutoff values were generally <0.1). (B) Competition ELISA showing that serum N1 has reactivity specific for the Palo Alto repeats and very low cross-reactivity to the 3D7 repeats. (C) Competition ELISA showing that serum N20 has predominant reactivity to the 3D7 repeats and less reactivity to epitopes that are on the full-length antigens and on the Palo Alto repeats.

of the antigens to compete (examples for sera N20 and N15 are shown in Fig. 3B and C, respectively).

Reactivities of IgG in sera from African children to MSP1 block 2 repeat and flanking sequence antigens. The MSP1 block 2 repeat and flanking sequence antigens were tested with sera from 334 Gambian children who were between 3 and 7 years old and were sampled shortly before the start of the annual malaria transmission season in 1996. These sera had previously been studied for reactivity to full-length MSP1 block 2 antigens (14). The overall patterns of reactivities to MSP1 block 2 antigens were similar to those seen in the Nigerian

adult sera. Specific antibodies to each of the repeat antigens and the K1-like flanking sequence were commonly detected, but there was little reactivity with the MAD20-like flanking sequence. Figure 4 shows the levels of absorbance (optical density) for IgG reactivity in all sera with all tested repeat antigens, flanking sequence antigens, and representative full-length antigens for each of the K1-like and MAD20-like block 2 types.

Correlations between the reactivities to pairs of antigens were analyzed (Fig. 4). There were moderately strong correlations between the reactivities of the full-length antigens and

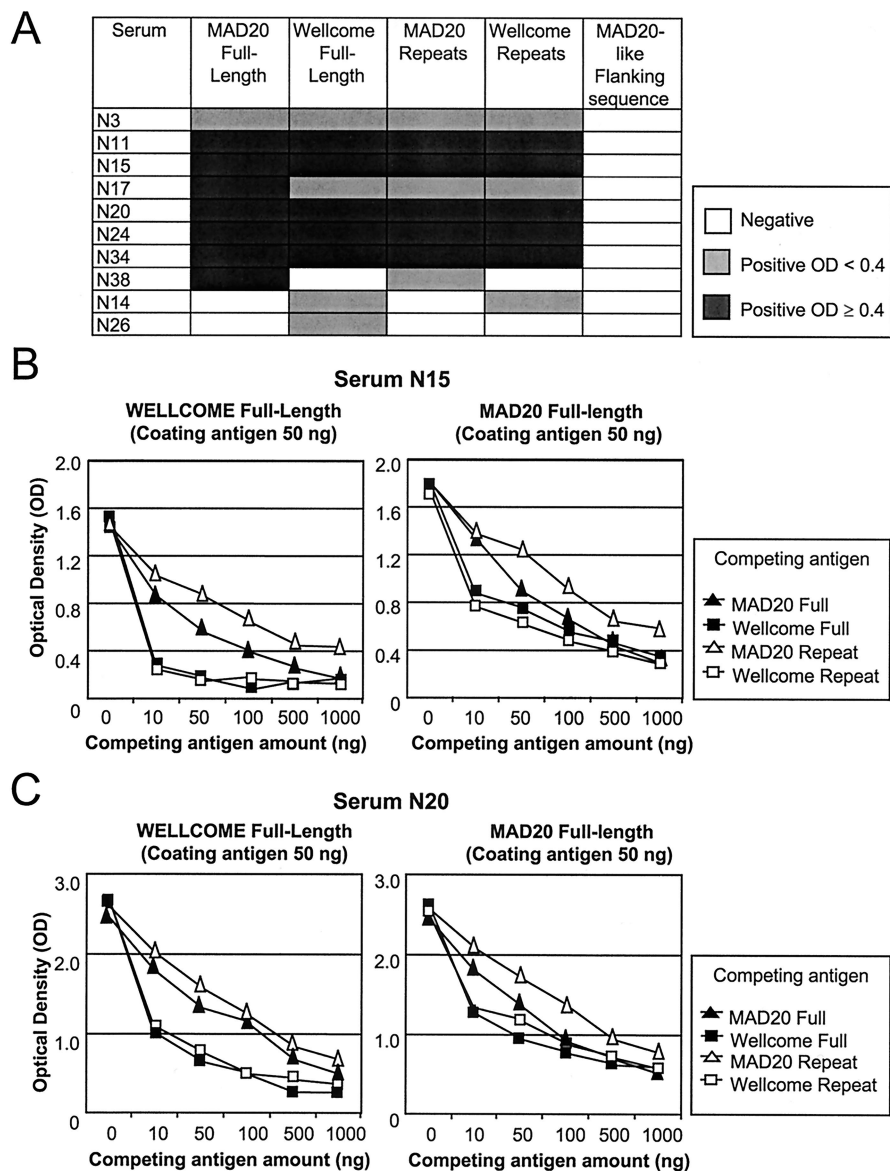


FIG. 3. Reactivities of Nigerian adult sera with MAD20-like antigens. (A) Reactivities of all sera positive for one or more MAD20-like antigens. Positive ELISA optical density (OD) values are the values above the cutoff defined for each antigen by the mean plus 3 standard deviations for negative control sera (the cutoff values generally were <0.1). (B) Competition ELISA showing that serum N20 has reactivity to epitopes shared by the MAD20 and Wellcome repeats and that the Wellcome repeats have greater competitive binding ability. (C) Competition ELISA showing that serum N15 similarly has reactivity with epitopes shared by the MAD20 and Wellcome repeats. Again, the competitive binding by Wellcome repeats is greater.

the corresponding repeats for the K1-like antigens ($r = 0.53$ for Palo Alto repeats versus Palo Alto full-length antigen; $r = 0.70$ for 3D7 repeat versus 3D7 full-length antigen) (Fig. 4A). The correlations between the reactivities of full-length and repeat MAD20-like antigens were stronger ($r = 0.86$ for MAD20

repeat versus MAD20 full-length antigen; $r = 0.92$ for Wellcome repeat versus Wellcome full-length antigen) (Fig. 4B). Strong correlations for reactivities to K1-like flanking sequence and K1-like full-length antigens were also seen ($r = 0.75$ for Palo Alto; $r = 0.62$ for 3D7) (Fig. 4C). In contrast,

FIG. 4. Correlation of reactivities of sera from Gambian children who were 3 to 7 years old ($n = 334$) with pairs of recombinant proteins containing different MSP1 block 2 sequences. Examples are shown for K1-like block 2 type proteins in panels A, C, and E and for the MAD20-like block 2 type proteins in panels B, D, and F. Absorbance (optical density) values for IgG reactions with each antigen were plotted for pairwise combinations of antigens, as follows: repeats versus full-length antigens (A and B); flanking sequences versus full-length antigens (C and D); and different repeat sequences within a type (E and F). The dashed lines indicate the cutoff values for positive reactions (defined as the mean plus 3 standard deviations for European control values). Pearson's correlation coefficients (r values) are shown.

K1-like block 2 type

MAD20-like block 2 type

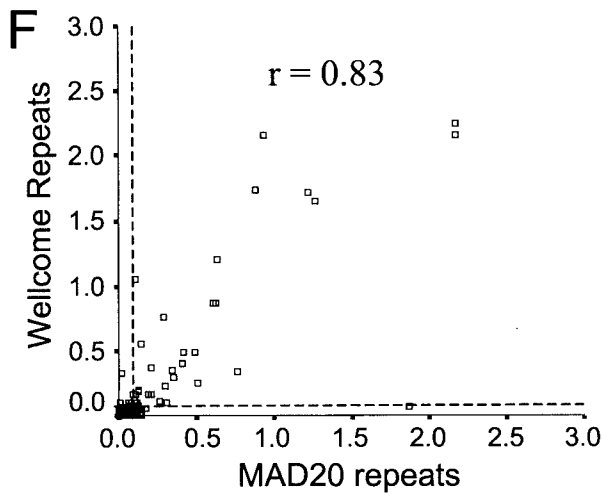
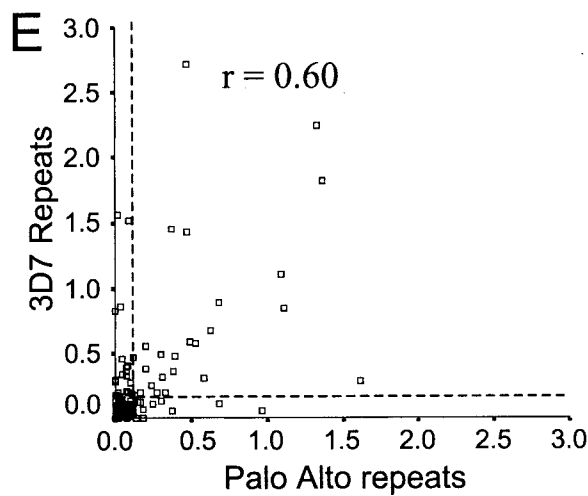
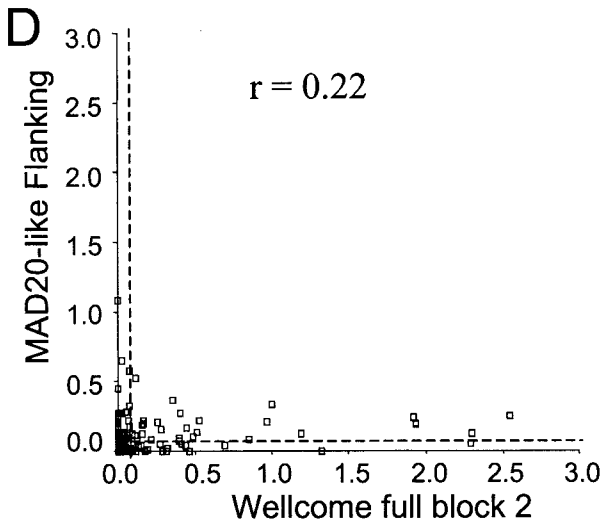
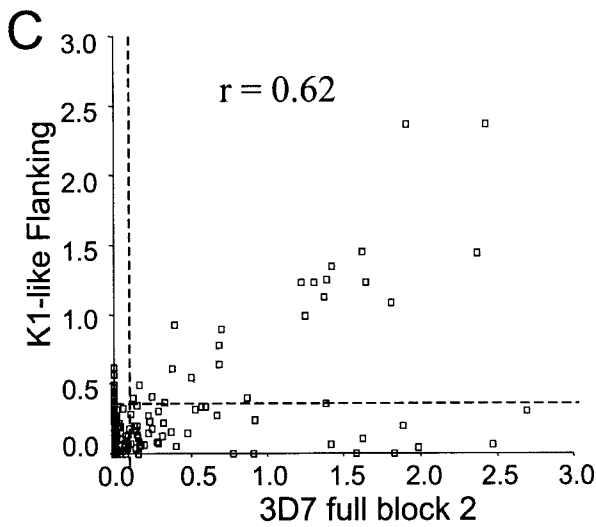
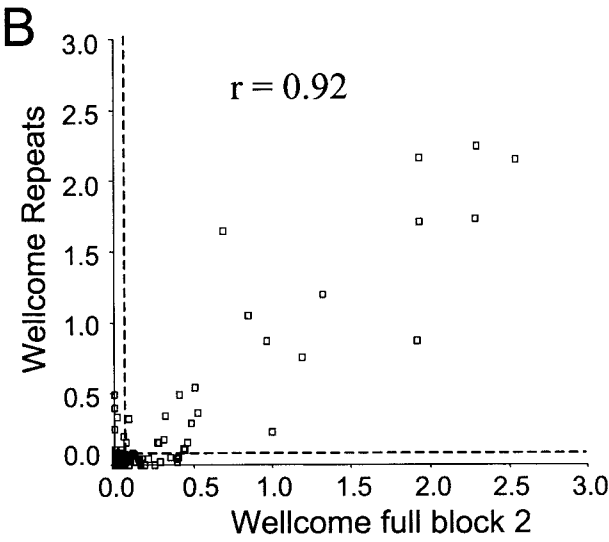
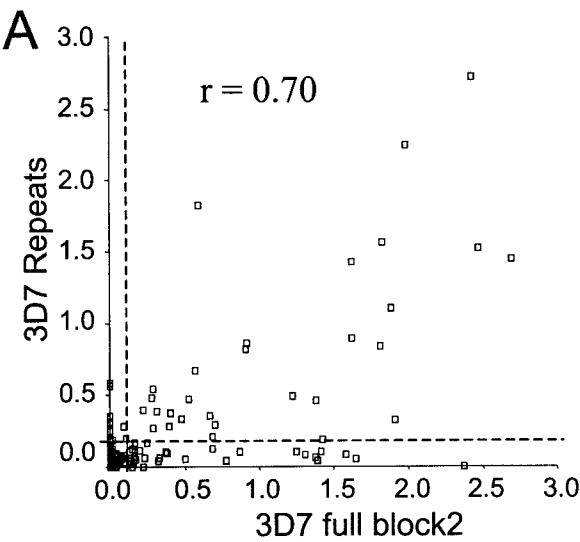


TABLE 1. Proportions of Gambian children acquiring clinical malaria in July to November in groups with or without serum IgG to different parts of MSP1 block 2 in June

| MSP1 recombinant antigen | % Acquiring clinical malaria ^a | | Relative risk (95% confidence interval) | Univariate <i>P</i> value | Age-corrected <i>P</i> value |
|--------------------------|---|--------------------------|---|---------------------------|------------------------------|
| | IgG-positive individuals | IgG-negative individuals | | | |
| K1-like block 2 | | | | | |
| Flanking nonrepeats | 46.9 (15/32) | 55.0 (166/302) | 0.85 (0.58–1.25) | 0.38 | 0.55 |
| Palo Alto repeats | 27.3 (9/33) | 57.1 (172/301) | 0.48 (0.27–0.84) | 0.001 | 0.014 |
| 3D7 repeats | 32.4 (11/34) | 56.7 (170/300) | 0.57 (0.35–0.94) | 0.007 | 0.047 |
| MAD-like block 2 | | | | | |
| Flanking nonrepeats | 56.8 (42/74) | 53.5 (139/260) | 1.06 (0.84–1.33) | 0.62 | 0.36 |
| Wellcome repeats | 32.5 (13/40) | 57.1 (168/294) | 0.57 (0.36–0.90) | 0.003 | 0.012 |
| MAD20 repeats | 41.3 (19/46) | 56.3 (162/288) | 0.73 (0.51–1.05) | 0.059 | 0.192 |

^a A total of 334 children were studied. The values in parentheses are number who acquired clinical malaria/total numbers who were IgG positive or IgG negative for the given antigen.

there were only weak antibody reactivities to the MAD20-like flanking sequence protein, and these reactivities did not correlate well with antibody reactivities to the full-length antigens ($r = 0.30$ for the correlation with MAD20 full-length block 2; $r = 0.22$ for the correlation with Wellcome full-length block 2) (Fig. 4D). The lower correlations between the reactivities of the K1-like full-length and repeat antigens than between the reactivities of the MAD20-like full-length and repeat antigens can be explained by the sera that bind only to the K1-like flanking sequence. No serum that had strong reactivity to a K1-like full-length antigen failed to recognize either the repeats or the flanking region (sera represented by points at the bottom right of the scatter plot in Fig. 4A are represented by points at the top right of the scatter plot in Fig. 4C and vice versa).

There was a very strong correlation between the reactivities to the two MAD20-like repeat antigens (MAD20 and Wellcome; $r = 0.83$) (Fig. 4F), suggesting that a high proportion of antibodies react with both. This is consistent with the cross-reactivities in sera of Nigerian adults as determined by competition ELISAs. For the K1-like repeat antigens (Palo Alto and 3D7), the correlation was lower ($r = 0.60$) (Fig. 4E), suggesting that a high proportion of antibodies against K1-like repeats recognize subtype-specific epitopes. This is also consistent with the reactivities in Nigerian adult sera revealed by competition ELISAs.

Relationship between antibodies to MSP1 block 2 repeat and flanking sequence antigens and subsequent incidence of malaria. The IgG reactivities to each of the block 2 antigens in the Gambian children in June prior to the malaria transmission season were tested for associations with the subsequent incidence of clinical malaria in the children throughout the malaria transmission season (July to November). Table 1 shows that the presence of IgG to the Wellcome, 3D7, and Palo Alto repeats was significantly associated with a lower risk of clinical malaria (the relative risk values were significantly less than 1.0). These protective associations remained statistically significant when age was accounted for as a confounding variable by multivariate (logistic regression) analysis. Inclusion of other variables (such as the presence of *P. falciparum* parasitemia at the time of serum sampling prior to the transmission season) in multivariate analysis did not alter these associations. The presence of IgG to the MAD20 repeats showed a nonsignificant

trend towards protection. When the optical densities (rather than just positive reactions) for the MAD20 repeats were analyzed, there was a significant correlation with protection ($P = 0.014$, as determined by univariate analysis; $P = 0.026$ after adjustment for age, as determined by multivariate analysis). The antibody reactivities to K1-like or MAD20-like flanking sequence proteins were not associated with protection, based on analysis of either antibody positive reactions or optical densities.

The presence of antibodies to different repeat sequences was associated with protection in an additive manner (Table 2). Individuals with antibody reactivity to only one or two of the different repeat antigens did not have a significantly lower risk of malaria than individuals with no antibodies. However, individuals with antibodies to three or more of the repeat antigens had a very significantly lower risk of malaria (relative risk, 0.20; 95% confidence interval, 0.06 to 0.75; $P = 0.0002$). This was particularly due to the fact that individuals had antibodies to repeat antigens of both K1-like and MAD20-like types. For example, individuals with antibodies to both the 3D7 repeats (K1-like type) and the Wellcome repeats (MAD20-like type) had a very low prospective risk of clinical malaria, corresponding to a relative risk of 0.26 (95% confidence interval, 0.06 to 0.77), compared with individuals who had neither ($P = 0.003$, as determined by univariate analysis; $P = 0.008$ after adjustment for age, as determined by multivariate analysis). Similarly, individuals with antibodies to both the Palo Alto repeats (K1-like type) and the MAD20 repeats (MAD20-like type) had

TABLE 2. Serum IgG reactivity to multiple MSP1 block 2 repeat antigens before the transmission season is associated with protection in an incremental manner

| No. of repeat antigens recognized | No. of sera | No. (%) of individuals getting malaria | Relative risk (95% confidence interval) | <i>P</i> value |
|-----------------------------------|-------------|--|---|----------------|
| 0 | 253 | 146 (58) | 1 | |
| 1 | 38 | 22 (58) | 1.00 (0.75–1.34) | 0.98 |
| 2 | 26 | 11 (42) | 0.73 (0.46–1.16) | 0.13 |
| 3 | 5 | 0 (0) | 0 (∞) | 0.015 |
| 4 | 12 | 2 (17) | 0.29 (0.08–1.03) | 0.005 |
| 3 or more | 17 | 2 (12) | 0.20 (0.06–0.75) | 0.0002 |

a very low prospective risk of clinical malaria, corresponding to a relative risk of 0.37 (95% confidence interval, 0.13 to 1.01), compared with individuals who had neither ($P = 0.007$, as determined by univariate analysis; $P = 0.039$ after adjustment for age, as determined by multivariate analysis).

DISCUSSION

In this study we used new recombinant proteins to dissect the structure of *P. falciparum* MSP1 block 2 and to map the specificity of antibodies associated with protection in humans. The results have implications for vaccine design because they demonstrate the importance of the block 2 polymorphic repeat sequences in naturally acquired immunity and provide an assessment of the cross-reactivity of human antibodies to these sequences.

Previously, immunization of mice with full-length block 2 antigens resulted in mainly type-specific IgG responses (10), and type specificity was also predominantly seen here in response to MAD20-like repeat sequences (antibodies recognizing both Wellcome and MAD20 repeats) and the K1-like flanking sequence. However, the responses to the K1-like repeats were mainly subtype specific (discriminating between Palo Alto and 3D7 repeats), indicating that these two repeats are more antigenically distinct, as expected from their primary sequences (Fig. 1). Immunization with the MAD20-like flanking sequence protein elicited IgG with a low titer or no reactivity against parasites as determined by IFA, indicating either that B-cell epitopes are not present in the sequence or that the recombinant protein does not reflect the native structure of such epitopes.

Human sera from individuals in malaria-endemic populations in Nigeria and The Gambia showed clear and consistent patterns of reactivities with the MSP1 block 2 recombinant proteins. For the MAD20-like antigens, type-specific antibodies were seen in the majority of positive sera and reacted with sequences common to the Wellcome and MAD20 repeats. No or very weak reactivities with the MAD20-like flanking sequence protein were the rule, which is consistent with the observation that almost all antibodies against MAD20-like antigens were directed against the repeats. We cannot eliminate the possibility that there might be epitopes in the flanking sequence that were not represented by the recombinant flanking sequence protein, although if such epitopes exist, they must be rarely recognized. With K1-like proteins, type-specific antibodies against the flanking sequences were detected, and antibodies against the repeats were more predominantly subtype specific (discriminating between Palo Alto and 3D7).

The strong associations between reactivity of antibodies to the individual MSP1 block 2 repeat antigens and a lower prospective risk of clinical malaria shown here indicate that the repeat sequences contain protective antibody epitopes. It is important that these protective associations remained significant after adjustment for age of individuals and the presence of pretransmission season parasitemia. In addition to the protective associations of antibodies to each of the repeat antigens separately, there was an additive effect. In particular, the individuals with reactivities to repeats of both types (K1-like and MAD20-like) were much less likely to have malaria during follow-up. It has recently been shown that the human antibodies to MSP1 block 2 are predom-

inantly IgG3 (12, 20), a cytophilic subclass implicated in protection from malaria (7). Antibody responses to MSP1 have been shown to be short-lived in some studies (11, 17), and it has been proposed that the short half-life of serum IgG3 may contribute to this (12). Such antibodies could have a transient effect in controlling existing parasitemia but might not provide long-term protection against subsequent infections. The length of time that antibody levels last after infection is therefore an important area for further investigation, and there may be differences among populations (20).

It has been suggested that there may be cross-reactivities between B-cell epitopes on different repeat structures of *P. falciparum* antigens and that these cross-reactivities could prevent the development of effective antibodies (2) by interfering with affinity maturation of B cells. In contrast, results obtained in this study show that antibodies to the different MSP1 block 2 repeats are highly specific and not cross-reactive between the major allelic types (K1-like and MAD20-like) or even between certain subtypes of the K1-like sequences. In another hypothesis workers have proposed that immunodominant repeats could divert the human immune response away from more important targets of immunity (a distraction which has been likened to a smoke screen) (2, 9). However, the highly significant association between antibodies to the MSP1 block 2 repeats and the reduced risk of malaria shown in this study is evidence that B-cell responses to these repeats are effective in immune protection. Thus, the data do not support the inhibition of affinity maturation hypothesis or the immunodominant smoke screen hypothesis for the repeats of MSP1 block 2.

Rational vaccine design requires a comprehensive understanding of the repertoire of naturally occurring antigenic structures and evaluation of whether naturally occurring immune responses are protective. It is clear that any vaccine based on MSP1 block 2 sequences needs to protect against the whole spectrum of parasites with the divergent MSP1 block 2 sequences that occur naturally (24). Such a vaccine should include repeat motifs from MAD20-like and K1-like types in order to generate antibodies with specificities similar to those described here. A third allelic type (the RO33-like type, which does not contain repeats) requires further study as antibodies against this type have not yet been associated with protection (14), although asymptomatic infection by parasites with the RO33-like allele has been associated with a reduced risk of clinical malaria (9). It will be important to further characterize the fine specificity of antibodies to each of these types and to determine whether these antibodies are associated with protection from malaria in other populations.

Effector mechanisms of antibodies against MSP1 are primarily thought to involve inhibition of erythrocyte invasion by merozoites (23, 25, 34), but a potential role of monocytes in antibody-dependent inhibition of parasite growth may also be important (6). To elicit lasting antibody responses by immunization, helper T-cell responses are also required. T-cell reactivities to MSP1 block 2 have not been identified yet, but T-cell epitopes in proximal sequences of block 1 (27, 28) are promising candidates for inclusion with block 2 antibody epitope sequences in an experimental vaccine. Other aspects of formulation, including potential combination with C-terminal MSP1 sequences, require downstream empirical investigation.

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